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VALIDATION OF A PARAOXON-BASED METHOD FOR MEASUREMENT OF PARAOXONASE (PON-1) ACTIVITY AND ESTABLISHMENT OF REFERENCE INTERVALS IN HORSES

Short title: PON-1 activity in healthy horses and foals

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Abstract

Background: Paraoxonase-1 (PON-1) is an anti-oxidant compound that is considered a negative acute phase protein ~~in animals and people~~. No information on the analytical performance~~s~~ of the paraoxon method for measuring PON-1 in horse serum is available.

Objectives: The aim of this study is to validate a paraoxon-based method to measure PON-1 in horses and to establish Reference Intervals (RIs) in healthy horses and foals.

Methods: One hundred and twenty horses and 55 foals classified as healthy after physical examination and routine biochemistry were used in the study~~We considered 120 horses and 55 foals classified as healthy after physical examination and routine biochemistry~~. Serum PON-1 activity was measured with an automated spectrophotometer and an enzymatic method validated in other species. After the analytical validation (precision, accuracy, interference studies), RIs were determined using the Reference Value Advisor software. The possible gender-, age- and breed-related differences were statistically investigated.

Results: The paraoxon-based method was precise (CVs <4.0%) and accurate (P<0.001 in linearity under dilution and spike-recovery testing) but is affected by interference from mild bilirubinemia, severe lipemia or hemoglobinemia. The RIs recorded in the whole population was 38.1-80.8 U/mL. According to the Harris and Boyd test, it would be advisable to use separate RIs only for adult females and for Warmblood and Trotter adults.

Conclusions: This study demonstrated that the analytical performances of the paraoxon-based method for measurement of PON-1 in horses are acceptable. PON-1 activity is lower in horses than in other domestic species. These results may provide a basis for further studies designed to establish whether healthy and sick horses can be correctly classified by using the PON-1 assay.

Keywords

Acute phase proteins, equine, inflammation, oxidative stress

Introduction

Inflammation is characterized by oxidative phenomena and oxidative stress (OS) is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants.

The serum activity of paraoxonase-1 (PON-1) decreases during inflammation in many species.[1-3] PON-1 is an enzyme associated with high-density lipoproteins (HDL) that protects low-density lipoproteins (LDL) and HDL from peroxidation.[4] Moreover, PON-1 possesses anti-inflammatory properties, as it reduces the production of pro-inflammatory mediators.[5] PON-1 is mainly expressed in the liver and is transported in the plasma bound to HDL. During an acute phase response, HDL molecules lose apolipoprotein A1, esterified cholesterol, and HDL-associated enzymes, including PON-1, which is replaced mainly by serum amyloid A and ceruloplasmin. Altogether, these phenomena result in reduced anti-oxidative properties of HDL.[6]

In both laboratory animals[7,8] and people,[9] these changes in HDL composition and structure during an acute phase response, inactivate the PON-1 and, in addition, the hepatic gene expression of PON-1 is inhibited.[7,9] For these reasons, PON-1 is considered a negative acute phase protein (APP).

In people, PON-1 is a potential biomarker for many pathological conditions, such as cardiovascular diseases, neurological disorders, and liver diseases.[10]

Despite the extensive use of PON-1 as a marker of inflammation/oxidation in people and lab animal species, its use in veterinary medicine is limited. Changes in PON-1 activity have been investigated in cattle,[2,11,12] cat,[13] swine[3] and dog[1,14-16] but using different methods and without

a complete preliminary validation study or the establishment of reference intervals (RIs). So far, the paraoxon-based method to measure PON-1 activity in serum has been validated only in dog[1] and cattle.[2]

Method validation guarantees the reliability (precision) and validity (accuracy) of analytical results, especially when a new method or analyte is introduced and is a key component of quality management.[17]

According to ASVCP guidelines,[18] RIs are ~~nowadays~~ an integral component of laboratory diagnostic testing and clinical decision-making and represent estimated distributions of reference values from healthy populations of comparable individuals.[19] Clinicians convert the results provided by the laboratory into information of diagnostic, prognostic, or therapeutic relevance. Inappropriate RIs could, therefore, lead to erroneous and delayed clinical decisions.[20]

A minimum of 120 reference individuals is recommended in order to determine reference limits by nonparametric methods with 90% confidence intervals (CI). However, smaller sample sizes may be used if appropriate statistical methods are employed.[21] In the establishment of RIs, it is also important to take into account any potential source of biological variability such as age, gender, and/or ~~aptitude/~~use.[18,20,21] This may be particularly important in young animals that, in other species, have been demonstrated to have a lower PON-1 activity.[2]

The aims of this study are to validate a paraoxon-based method to measure PON-1 in horses and to define RIs in healthy horses and foals as a preliminary step towards the future use of PON-1 in clinical practice.

Materials and methods

Case selection

One hundred and twenty horses (40 geldings, 40 stallions and 40 mares) and fifty-five foals (27 females, 28 males) were included in this study. The median age in adult horses was 11 years (age range 3-27 years) without significant differences between the age distribution of females (median age: 11 years; age range 4-21 years), entire males (median age: 8 years; range: 3-27 years) and geldings (median age: 14.5 years; range: 4-25 years). The median age in foals was 47 days (range 19-90 days), without significant difference in the age distribution between females (median age: 50 days; range 19-90 days) and males (median age: 46 days; range: 29-90 days). Horses and foals were grouped by breed: Thoroughbreds (6 adult horses), Trotters (31 foals and 46 adult horses), Warmbloods (22 foals and 57 adult horses), Draft horses (3 adult horses) and Ponies (8 adult horses).

Horses and foals were classified as clinically healthy on the basis of physical examination and routine biochemistry and hematology. Horses with particular pathophysiological conditions (pregnancy, lactation, obesity) were not included in the study. Samples were collected between March and June and horses were not fasted ~~at the moment of~~before sampling. Blood (10 mLs) was collected from the jugular vein into tubes without anticoagulant (Venosafe plastic tubes for serum, Terumo, Europe), was centrifuged within 4 hours of collection and serum was frozen at -20 °C until analysed. Serum PON-1 was measured at the Department of Veterinary Medicine, University of Milan.

The study was performed within plans of health monitoring and the protocol was approved by the Ethical Committee of the University of Pisa [prot. n. 23506/16]. An owner's written consent was also signed.

Measurement of serum PON-1 activity

Serum PON-1 activity was measured spectrophotometrically using an automated analyser (Cobas Mira, Roche diagnostic, Basel. Switzerland), using the enzymatic method previously described[7] and already validated in dogs[1] and in cattle.[2] Briefly, 6 μL of serum was incubated at 37°C with 89 μL of distilled water and 100 μl of reaction buffer (glycine buffer 0.05 mM, pH 10.5 containing 1 mM of paraoxon-ethyl, purity > 90% [Sigma-Aldrich, Saint Louis, MO, USA], and 1 mM of $CaCl_2$). The rate of hydrolysis of paraoxon to p-nitrophenol was measured by monitoring the increase in absorbance at 504 nm using a molar extinction coefficient of 18,050 L· mol-1 · cm-1. The unit of PON activity expressed as U/mL is defined as 1 nmol of p-nitrophenol formed per minute under the assay conditions.

Analytical validation

The intra-assay precision was determined by measuring the PON-1 activity in pooled equine sera with low, medium and high PON-1 activity 20 consecutive times within a single run of analysis.[22,23] The inter-assay variability was assessed on frozen aliquots by analysing the same samples in triplicate on 10 consecutive working days.

The mean value, the standard deviation (SD), and the coefficient of variation (CV = SD/mean x 100) were then calculated.

The accuracy was determined using the evaluation of linearity under dilution (LUD) and a spiking recovery test (SRT). The LUD test was performed by measuring PON-1 activity in triplicate on a pooled equine serum after dilution with distilled water to obtain samples containing 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 0% of serum. The SRT test was performed by adding a pooled equine serum with low PON-1 activity with increasing volumes of a pooled equine serum with high PON-1 activity, followed by the measurement of PON-1 activity in triplicate.

The correlation between the percentage of recovery compared with expected values of LUD and SRT tests were assessed using a least square regression test.

Interference studies

Five serum samples from control horses that at macroscopical examination appeared non-hemolytic, non-lipemic and non-hyperbilirubinemic were pooled in order to obtain a sufficient volume to create multiple aliquots to be tested with the different interfering substances. The effect of hemolysis, hyperbilirubinemia and hyperlipemia was assessed using previously published protocols.[1] Specifically, the pooled sample ~~was added with~~had different concentrations of HGB (Merck KGaA, Darmstadt, Germany), bilirubin (Bil) (Fluka, Sigma-Aldrich, Milano, Italy), or a commercial fat emulsion (Trig) (Lipofundin S 20%, B. Braun Milano SpA, Milano Italy) added, followed by triplicate measurement of PON-1 activity. Interfering substances were added to each aliquot of the pooled sample in order to obtain final concentrations adequate to simulate the following levels of hemoglobinemia, lipemia and icterus: slight (HGB = 1,25 g/L; Trig = 1.25 g/L; Bil = 0.12 mg/L), moderate (HGB = 2,5 g/L; Trigl = 2.5 g/L; Bil = 0.25 g/L, severe (HGB = 5,0 g/L; Trigl =5.0 g/L; Bil = 0.50 g/L), and extreme (HGB = 10.0 g/L; Trigl = 1.0 g/L; Bil = 0.15 g/L).

To further investigate the effect of hemolysis, a hemolysate of equine erythrocytes was also added to the pooled serum~~To further investigate the effect of hemolysis we also added the pooled serum with a hemolysate of equine erythrocytes~~.[24] The rationale for using this approach is to assess the influence of intraerythrocytic compounds that could theoretically interfere with PON-1 activity (e.g. intraerythrocytic enzymes) or with PON-1 measurement (e.g. intraerythrocytic cations that could interfere as cofactors of the in vitro reaction used to measure PON-1 activity). To this aim, equine blood samples collected in EDTA and submitted

to the diagnostic laboratory of the Department for routine hematology were centrifuged at 2500 G x 10 min. The pellet obtained after removal of plasma, was then washed twice with phosphate buffered saline (PBS) in order to completely remove plasma. After the second wash and a further centrifugation, PBS and buffy coat were removed by aspiration and a hypotonic lysis of RBCs was then performed by adding and equal volume of distilled water to the cell pellet, followed by ~~further by adding the RBC pellet with an equal volume of distilled water and further~~ centrifugation~~ed~~. The final concentration of HGB in the supernatant was verified by a hematology analyser (Sysmex XT-2000iV, Sysmex Corporation, Kobe, Japan). Based on this concentration, the pooled serum was added with the hemolysate to obtain a final concentration of HGB of 10.0, 5.0, 2.5 and 1.0 g/L thus simulating a rate of hemolysis accounting for about 1% to about 7% of the mean RBC mass of a normal equine blood sample.

For all the interferents, the percentage changes of PON-1 activity, compared with the basal sample, with the same volume of distilled water as the volume of interfering solution being added to the serum, were calculated and plotted versus the concentration of interfering substances to create an interferogram for each substance.

Results obtained from the pooled serum with and without the different concentrations of interfering substances were compared using an ANOVA test for repeated measurement (Friedman test). Irrespective of the results of statistical analysis, a ±15% variability of mean values was established as an acceptance criterion to assess the clinical utility of results of the interference study.

Determination of RIs

The RIs were determined using the Reference Value Advisor macroinstructions (freeware

v2.1; http://www.biostat.envt.fr/spip/spip.php?article63) for Excel (Microsoft Corp., Redmond, WA, USA), recently validated for use in veterinary laboratories.[211] The software performs tests of normality (Anderson–Darling with histograms and Q–Q plots and Box–Cox transformation). Following the CLSI recommendations,[25] the histogram of RI of PON-1 activity was examined for initial assessment of distribution and identification of outliers. Dixon's and Tukey's tests were used to identify the outliers, with Tukey's test more stringent than Dixon's test. According to the CLSI guidelines[25] the emphasis was to retain rather than delete outliers. Specifically, near outliers (i.e., values exceeding quartiles I or III minus or plus 1.5 × IQR) were classified as "suspected" and retained, while far outliers (i.e., values exceeding quartiles I or III minus or plus 3.0 × IQR), if any, were removed,

The RIs were calculated using the robust method on Box-Cox transformed data and 90% Confidence Intervals (CI) around the reference limits were determined using a non-parametric bootstrap method.[26]

The possible differences depending on gender, age and breed were investigated. Specifically, results obtained in foals and adult horses were investigated using a Mann Whitney U test. The same test was used to investigate the possible differences between male and female foals or in Trotter vs Warmblood horses either in foals or in adults. Results recorded in geldings, mares and stallions were compared to each other using a Kruskall-Wallis test followed by a Bonferroni post-hoc test. The same test was used to compare the results obtained in the different breeds of adult horses. The possible age-related differences were investigated, either in foals or in adult horses, using a regression analysis run in the Reference Value Advisor software cited above.

Results

Analytical validation

Results regarding intra- and inter-assay precision are reported in table 1. The CVs were lower than 4% for all the three levels of PON-1 in the pooled sera explored in this study.

Results regarding LUD and SRT are reported in figure 1. Both the tests fitted the linear model (r^2 0.98, P<0.001 for the LUD test; r^2 1.00, P<0.001 for the SRT).

Results regarding interference studies are reported in figure 2. A significant and progressive decrease of PON-1 activity was found in lipemic samples: values exceeded the acceptance criterion (± 15%) when the concentration of triglycerides was equal to or higher than 5 g/L. Mild bilirubinemia induced a significant increase of PON-1 activity compared with the baseline value, while the other concentrations did not affect the activity of PON-1. This increase, however, did not exceed the acceptance criterion. Both hemoglobin and hemolysates induced a progressive increase of PON-1 activity, that became significant and exceeded the acceptance criteria at values corresponding to very severe hemolysis.

Reference intervals

Details about all the RIs recorded in this study are summarized in table 2, and data distribution is reported in the supplementary figure S1, according with the current guidelines for establishment of RIs.[18]

The RI recorded in the whole population of horses was 38.1-80.8 U/mL. The RIs recorded in adults and in foals were very similar to each other and the Harris and Boyd test[26] did not indicate the need of establish separate RIs for adults and foals. Moreover, no significant differences were found between foals and adults (figure 3).

Partitioning by age did not reveal significant differences neither within the group of foals (P=0.180) or within the group of adult horses (P=0.949) (data not shown).

Results recorded in male foals were not significantly different (P=0.963, figure 3) from those recorded in female foals. Harris and Boyd test suggests that RI specific for male and female foals should not be calculated. Conversely, significant gender-related differences (P=0.010) were found in adults: specifically, results recorded in mares were significantly higher than those recorded in stallions and in geldings (Figure 3). Despite the fact that the RIs of females, entire males and geldings overlapped, the Harris and Boyd tests indicates that a gender-specific RI should be used for females, while a common RI could be used for geldings and entire males.

The analysis of data recorded in adult draft horses (mean ± SD: 45.5 ± 12.4; median: 38.5; I-III interquartile: 38.2-56.2), Ponies (59.5 ± 6.0; 60.7; 55.6-62.3), adult Thoroughbreds (60.0 ± 13.8; 60.1; 46.6-70.1), Trotters (59.3 ± 9.8; 61.6; 39.3-65.6) and Warmbloods (54.4 ± 12.9; 51.8; 32.7-61.6) did not reveal significant differences (P=0.083), but when the analysis was restricted to the two breeds represented by a sufficient number of animals (Trotter vs Warmblood horses) a significant difference was found (Figure 3). Also in this case, breed-specific RIs overlapped but the Harris and Boyd test suggests that RIs specific for Trotter or Warmblood horses should be used.

No significant differences were found between PON-1 activity recorded in Trotter foals compared with Warmblood foals (figure 3). Also in this case the RIs recorded in Trotter foals and in Warmblood foals overlapped. The Harris and Boyd's test suggests that RIs specific for Trotter or Warmblood foals should not be used.

Discussion

~~Although S~~several studies of PON1 in other species have been reported.[1-3,11-16] A single study in horses is available but no information on the analytical performances of the method

263 employed were provided.[27] Before investigating the differences between clinically healthy and
264 sick horses and foals, however, a validation study was required to determine the analytical
265 performances of the paraoxon-based method of measurement, that in other species has been
266 preferred to other substrates because it is cheap, rapid to perform and very precise and
267 accurate.[1]
268 With this study, we demonstrated that this method for the measurement of PON-1 is precise
269 and accurate also on equine serum. Specifically, both intra-assay and inter-assay imprecision
270 were lower than 4% that is considered acceptable for most biochemical analytes[28] and is
271 similar or, as regards inter-assay imprecision, lower than that recorded in dogs.[1] Currently a
272 gold standard method for the evaluation of PON-1 does not exist and this may be a limitation
273 of the study. Therefore, accuracy was indirectly estimated through the evaluation of linearity
274 under dilution and of a spiking-recovery test which both fitted the linear model confirming the
275 excellent level of accuracy of the paraoxon-based method also in horses.
276 Our study demonstrated that the interference of bilirubin is minimal and not clinically relevant.
277 This finding is consistent with that observed in dogs[1] and is extremely important in horses,
278 where hyperbilirubinemia may be a non-specific alteration associated with several
279 pathophysiological conditions.[29,30] Also the variations of PON-1 activity associated with
280 lipemia, hemoglobinemia or hemolysis are similar to those already recorded in dogs[1] and
281 therefore it would be advisable to not analyse PON-1 activity in samples with slight to very
282 severe lipemia or with very severe hemolysis, ion which PON-1 activity may falsely decrease
283 or increase, respectively.
284 Moreover, it was shown that in horses PON activity measured by the paraoxon-based method
285 employed in this study is lower than that of dogs, cats and bovine.[1,2,13] It could be
286 hypothesized that different species have a different hepatic or lipid metabolism that influences

PON-1 level or that every species has its own particular isoforms that reach different serum concentrations. In people, in fact, the polymorphism of selected regions of PON-1 genes may influence the types of enzymatic activity (e.g. esterase and/or lactonase) thus explaining the wide individual variability in term of capability to interact with different substrates in vitro.[31]

No significant differences were observed between values recorded in adult horses and in foals; this likely depends on the age of the foals. In other species, very low PON-1 activity was recorded only in newborns, with significant increases from day 3 to 21 after birth,[2] possibly due to the immaturity of the liver, to differences in lipid metabolism to a high susceptibilty of newborns to oxidative stress.[32] In our caseload, foals were sampled from 19 to 90 days of age, when, ~~also~~ as in calves, PON-1 activity was not significantly different from that of adults.[2] Hence, it would be advisable in the future to assess whether PON-1 activity is low in the first days of age also in horses. This information would be particularly interesting, since most of the inflammatory conditions that may be better diagnosed or monitored with this novel biomarker, such as neonatal septicemia, typically occurs a few days after birth.[33,34]

Partitioning by age did not show any significant association between PON-1 activity either in adult horses or foals. This conclusion is similar to that in studies of people, where no significant age-related differences were found.[35]

The detection of higher PON-1 activity in mares contrasts with findings in dogs[1] but is consistent with that reported in mice[36] and people, where the difference was significant in some studies,[37] but not in others[35] and where this difference may be associated with a genetic predisposition.[35,38-42]

Differences that we found by sex, however, were present only in adult horses and not in foals, maybe because foals were below the age of sexual maturity. This is consistent with previous studies in mice, that showed that gender-associated differences were testes but not plasma

testosterone dependent and that ovariectomy had no effect on PON-1 mRNA expression.[39] However, further studies are needed to fully assess the effect of sex in foals, since the number of cases per group was lower than 40 individuals, the minimum database recommended by the ASVCP guidelines.[18]

After grouping the horses based on the breed, we found a significant difference only between Trotters and Warmbloods, with higher PON-1 activity in Trotter compared with Warmblood horses. This difference, however, was recorded in adult horses but not in foals, although the lack of differences in foals may be due to the low number ~~foals. Although also in this case the lack of differences in foals may depend on the low number~~ of cases per group compared with the minimum values recommended by the ASVCP guidelines.[18] However the higher values recorded in adult Trotters may depend on training and exercise, that may induce oxidative phenomena.[42] No significant differences in PON-1 activity were found when other breeds were also included ~~when also other breeds were included~~ in the statistical comparison. The absence of significant differences for other breeds could be related to the small number of horses in these groups that may have induced a type II statistical artefact.[43] Further studies with a higher number of horses of each of these breeds could be useful to identify possible differences associated with the different uses.

Despite the significant differences associated with gender or use, the RIs recorded in the different groups of animals overlapped ~~to~~ with each other. Nevertheless, according to Harris and Boyd test, specific RIs in mares, Trotter and Warmblood horses should be established. However, these latter aspects need to be verified through additional studies on a larger caseload since the analysis of the reference ranges and of data distribution reveals that the RI generated in the whole population is narrower and has a higher upper reference limit compared with the RIs of stallions, geldings or Warmbloods. This is likely to be due to ~~This~~

likely depends on the low number of cases per group after partitioning by gender or use. Independently of the need to establish gender- or breed-associated RIs, it is worth noting that it is worth to note that the lower reference limit of the intervals was similar for all the categories of partitioning examined in this study. Considering that, in many species, patients with OS associated with inflammation have very low PON-1 activity compared with the lower reference limit,[2,16] it is thus advisable to use a single RI for stallions and geldings or for foals and adults.

In conclusion, this study demonstrated that the paraoxon-based method for measurement of PON-1 activity is precise and accurate in horses as in other species and that PON-1 activity is lower in horses than in many other domestic species. Despite the presence of some significant differences associated with gender (with higher values in mares) or breed (with higher values in Trotters), the lower reference limits of age-, breed- or gender-associated RIs are similar to each other and therefore in routine practice it would be advisable to use a single RI for young foals and adult horses, independently on their gender or use. If future studies also demonstrate that also oxidative stress associated with inflammation induces a significant decrease of PON-1 activity compared with the RIs in horses, the results of this study will be useful in clinical practice to correctly classify healthy and sick horses according to PON-1 values. More specifically, PON-1 could be used, as in human medicine, in situations that can induce an oxidative stress, such as sepsis, cardiovascular, renal or liver disease. Moreover, results of sequential samples collected after treatment will allow assessment of whether PON-1 is a good marker will allow to identify if PON-1 may work as a good marker of response to treatments as in other species.[15]

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Conflict of interest statement

Authors disclose no conflict of interest.

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476 **Table 1**. Results of intra- and inter-assay imprecision calculated ~~i~~n pooled sera with high,
477 medium and low paraoxonase-1 activity (U/mL).
478

		High	Medium	Low
Intra-assay	**Mean**	61.01	34.02	24.04
	SD	0.57	0.55	0.62
	CV	0.93	1.60	2.57
Inter-assay	**Mean**	62.44	33.71	23.23
	SD	1.64	1.34	0.68
	CV	2.62	3.98	2.91

479

480 **Table 2**. RIs established in this study for the whole population of adult horses, for foals or for the subcategories of adult
481 horses. All the values are expressed in U/ml. N= number of horses; SD= standard deviation; CI= confidence interval; Distr=
482 distribution; G= gaussian; NG = non Gaussian; NP= non parametric; s = suspected outlier; RT = Robust Box-cox transformed

	N	Mean	SD	Median	Min	Max	Outliers	RI	CI Lower limit	CI Upper limit	Distr	Analysis type
Whole population	175	56.6	11.2	56.0	32.6	92.3	2 s	38.1-80.8	32.6-38.6	74.5-92.3	G	NP
Total adult horses	120	56.6	11.7	56.6	32.7	92.3	2 s	38.0-81.2	32.7-38.5	74.5-92.3	G	NP
Stallions	40	54.3	11.7	51.5	38.2	89.6	1 s	38.4-87.7	37.6-40.2	78.9-100.0	G	RT
Mares	40	60.3	9.6	62.7	34.9	81.2	1 s	37.3-77.5	29.7-45.4	74.7-80.4	NG	RT
Geldings	40	55.3	13.3	53.7	32.7	92.3	0	33.2-87.1	30.5-36.7	78.6-95.9	G	RT
Adult	46	59.3	9.8	61.6	39.3	81.2	0	37.6-	32.5-	75.3-	NG	RT

Trotters								78.4	43.7	82.0		
Adult Warmbloods	57	54.4	12.9	51.8	32.7	92.3	2 s	25.8-78.9	21.0-31.2	72.3-85.1	NG	RT
Foals	55	56.5	10.0	55.2	32.6	84.7	0	38.2-79.9	35.0-41.2	73.7-84.2	G	NP
Male foals	28	56.7	8.8	55.8	32.6	70.2	0	35.7-73.2	29.5-42.3	68.5-76.7	G	RT
Female foals	27	56.6	11.3	51.1	41.0	84.7	0	39.4-92.7	37.8-42.3	75.4-111.5	NG	RT
Trotter foals	31	56.5	11.2	51.9	32.6	84.7	0	35.4-84.5	31.7-40.1	72.5-92.7	NG	RT
Warmblood foals	22	56.4	8.6	55.5	43.7	70.2	0	39.5-76.3	36.6-44.2	70.5-81.5	G	RT

Figure captions

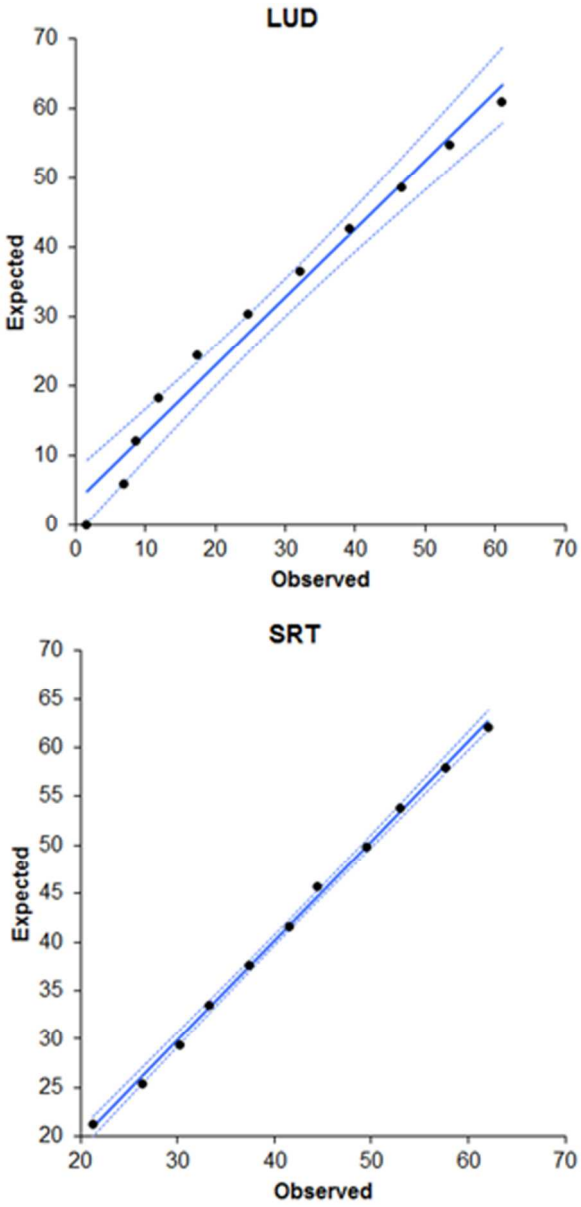
Figure 1. Linearity under dilution (LUD) of paraoxanase-1 (PON1) activity in a pool of equine sera (60.9 U/mL) progressively diluted (100% to 0%) with distilled water, and Spiking recovery test (SRT) of paraoxanase-1 (PON1) activity in a pool of equine sera with low PON1 activity (21.3 U/mL) spiked with increasing amounts of a pool of equine sera with high PON1 activity (62.0 U/mL). Each data point indicates the mean of a triplicate measurement. The solid line indicates the linear correlation between expected and observed values, dotted lines indicate the 95% Confidence Interval (CI).

Figure 2. Effects of increasing concentrations of interfering substances on PON-1 activity determined ~~on~~ in pooled equine sera (PON-1 activity of pooled serum: 59,9 U/mL). * = P<0.05 vs. baseline value (0 g/dL); ** = P<0.01 vs baseline value (0 mg/dL for bilirubinemia, 0 g/dL for hemoglobinemia and hemolysis); *** = P<0.001 vs baseline value (0 g/dL).

Figure 3. Comparison of results obtained in adult horses vs foals, stallions vs mares vs geldings, Trotters vs Warmblood adult horses, male vs female foals, and Trotter vs Warmblood foals. The boxes indicate the I–II interquartile range (IQR), the horizontal line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 × IQR or to further observation within quartile III plus 1.5 × IQR. '+' indicates near outliers (i.e., values exceeding quartiles I or III minus or plus 1.5 × IQR). The grey shaded area indicates the RI calculated for the whole population of horses. The asterisk indicates a significant difference (mares vs stallions and geldings; adult Trotters vs Warmbloods).

Supplementary material

Supplementary figure 1. Distribution of PON-1 values recorded in adult horses, foals and in the various categories of adult horses. The bars indicate the relative frequency of each unit of PON-1 activity. The pink line summarizes the fitted distribution. The vertical light blue lines indicate the upper and lower limit of the reference interval whereas the dashed lines indicate the 90% confidence intervals of each limit. A= whole population (adults and foals); B= adult horses; C= stallions; D= mares; E= geldings; F= Trotter (adults); G= Warmblood (adults); H= foals; I= male foals; J= female foals; K= Trotter (foals); L= Warmblood (foals).



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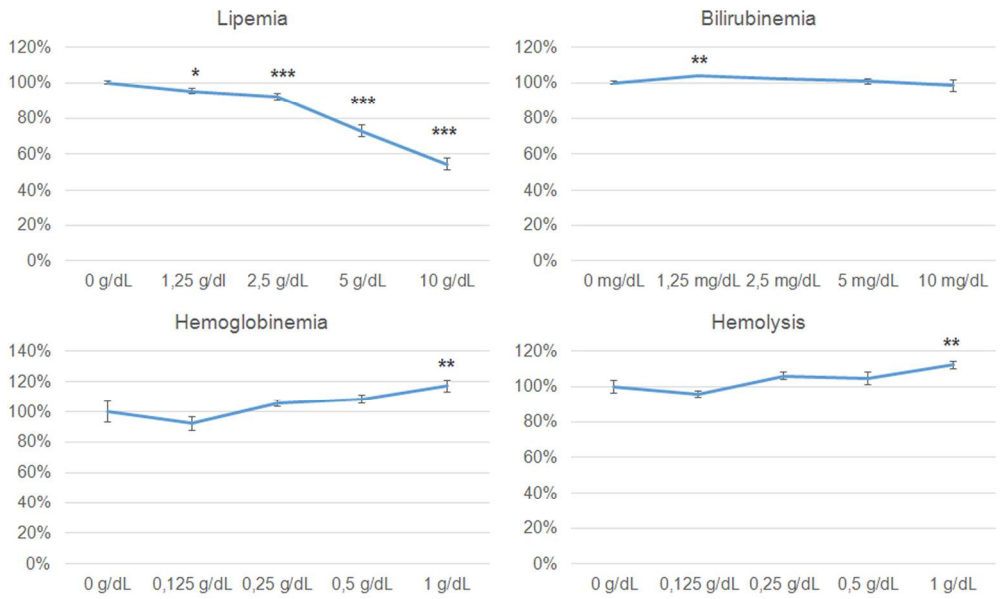

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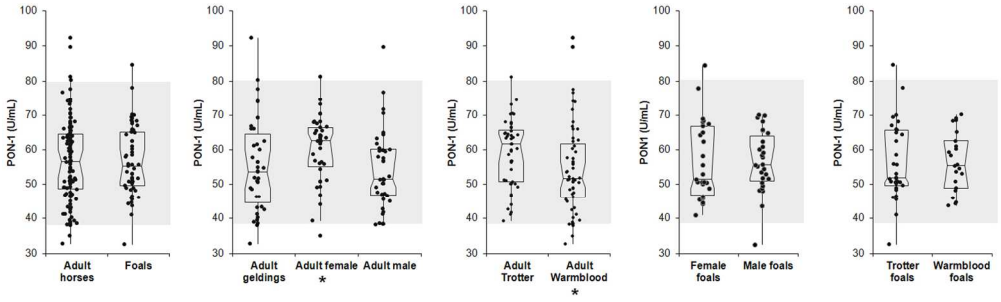

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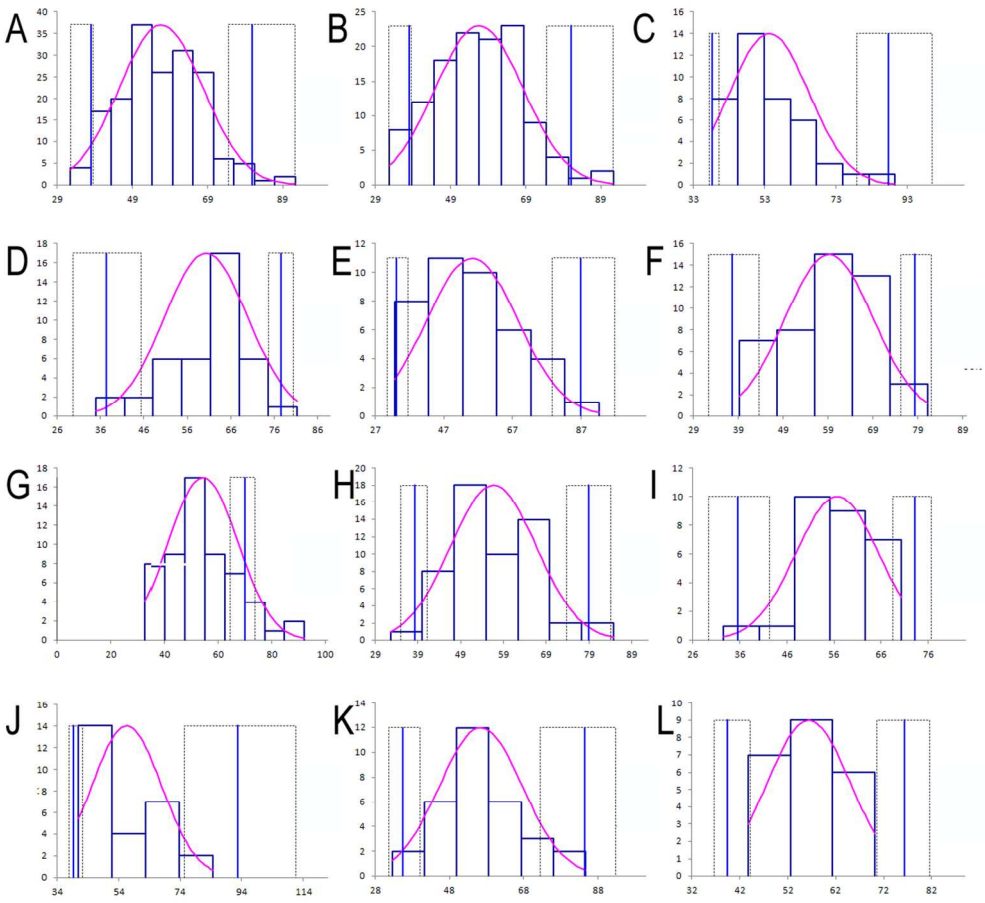
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